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Selective CD4$^+$ T Cell Help for Antibody Responses to a Large Viral Pathogen: Deterministic Linkage of Specificities

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SUMMARY

Antibody responses are critical components of protective immune responses to many pathogens, but parameters determining which proteins are targeted remain unclear. Vaccination with individual MHC-II-restricted vaccinia virus (VACV, smallpox vaccine) epitopes revealed that CD4$^+$ T cell help to B cells was surprisingly nontransferable to other virion protein specificities. Many VACV CD4$^+$ T cell responses identified in an unbiased screen targeted antibody virion protein targets, consistent with deterministic linkage between specificities. We tested the deterministic linkage model by efficiently predicting new vaccinia MHC II epitopes (830% improved efficiency). Finally, we showed CD4$^+$ T cell help was limiting for neutralizing antibody development and protective immunity in vivo. In contrast to the standard model, these data indicate individual proteins are the unit of B cell-T cell recognition for a large virus. Therefore, MHC restriction is a key selective event for the antiviral antibody response and is probably important for vaccine development to large pathogens.

INTRODUCTION

Vaccines are one of the most cost-effective medical treatments in modern civilization (Rappuoli et al., 2002). Vaccinia virus (VACV) is the viral species used as the human smallpox vaccine. The smallpox vaccine has been extraordinarily effective, having brought about the worldwide eradication of smallpox disease (Fenner et al., 1988). The smallpox vaccine is generally considered the gold standard of vaccines, and elucidating the immunobiology underlying the protection provided by the smallpox vaccine will continue to reveal vaccinology principles that can be applied to future vaccine development against other infectious scourges. However, identifying and analyzing the fine specificities of the adaptive immune response to a large pathogen—such as a poxvirus—is confounded by a number of factors, not least of which is the stark magnitudes of the potential peptide targets of the T cell responses and the potential protein targets of the antibody responses. As a result of these challenges, we possess only a piecemeal understanding of the fine specificities of T cell and antibody responses to any large pathogen and therefore have a thin understanding of the roles of each fine specificity in protective immunity, thereby limiting our ability to rationally design new vaccines against large and complex pathogens.

Although neutralizing antibodies are of primary importance in the protection from smallpox provided by the smallpox vaccine in animal models (Belyakov et al., 2003; Edghill-Smith et al., 2005; Galmiche et al., 1999; Lustig et al., 2005) and humans (Amanna et al., 2006; Demkowicz et al., 1992), CD4$^+$ T cells and CD8$^+$ T cells are also of great value (Amanna et al., 2006; Fang and Sigal, 2005; Tscharke et al., 2005; Xu et al., 2004). Here, we have focused on understanding the relationship between antibody and CD4$^+$ T cell responses to vaccinia virus in mice, as part of a strategy to elucidate the value of individual fine specificities, potential interrelationships between those specificities, and the underlying immunobiological and virological parameters that determine the emergence of protective immune responses to a small subset of all possible specificities.

RESULTS

Exquisitely Selective Antigen-Specific T Cell Help

Infection of mice with VACV results is an acute infection characterized by several days of high viral replication and viral loads of >10^8 infectious virions; this is followed by a strong adaptive immune response and viral clearance in 1–2 weeks (Amanna et al., 2006; Harrington et al., 2002; Xu et al., 2004). IgG responses to VACV are fully dependent on CD4$^+$ T cell help, as shown by the absence of VACV IgG in MHC-II-deficient mice (Figure 1A and Xu et al. [2004]). We recently identified 14 VACV MHC II epitopes after VACV infection of B6 mice (Moutaftsi et al., 2007). CD4$^+$ T cells of each specificity expressed CD40L after stimulation with cognate peptide (Figure 1B), indicating their competence to provide B cell help. In an effort to boost the antiviral antibody

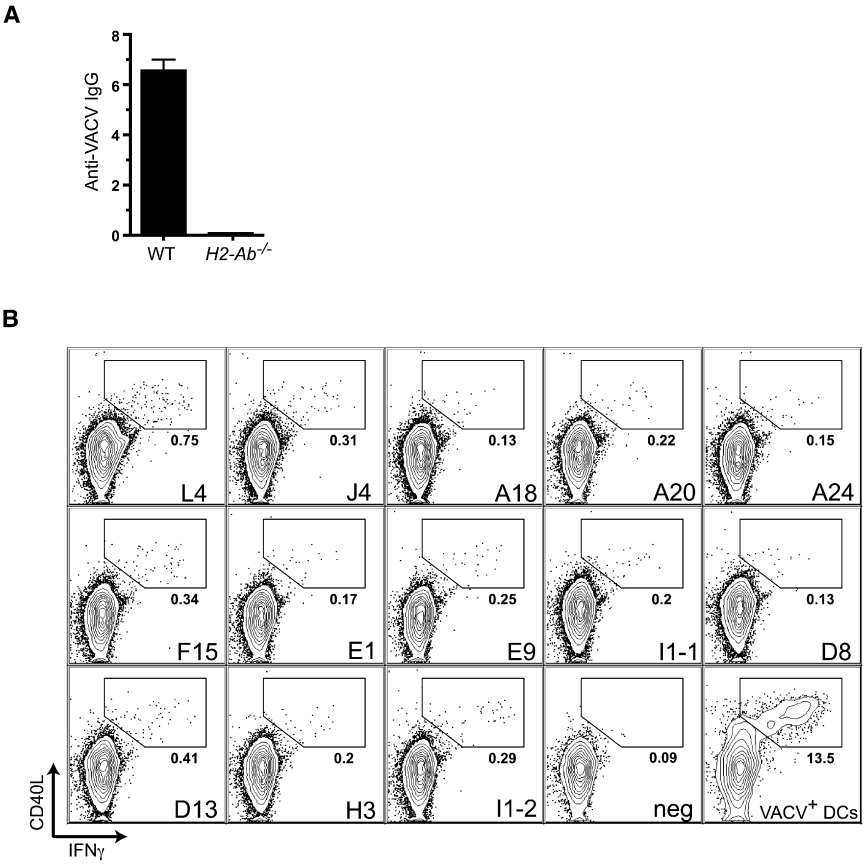

Figure 1. Vaccinia Virus CD4+ Helper T Cells and Helper T Cell Dependent Antibodies

(A) Quantitative ELISA of anti-VACV IgG (μg/ml), day 30 after infection in wild-type (WT) and MHC II-deficient (*H2-Ab*$^{-/-}$) mice. The graph shows mean ± SEM.

(B) Splenocytes from day 10 $VACV_{WR}$-infected mice were incubated with $CD11c^+$ dendritic cells (DCs) pulsed with VACV peptides (L4, J4, etc., names corresponding to the VACV protein from which each peptide epitope was derived). Cells were incubated for 6 hr and then stained for intracellular IFNγ and CD40L. Gated $CD4^+$ $CD62L^{lo}$ lymphocytes are shown, and percentages quantified are $IFN\gamma^+$ $CD40L^+$ of $CD4^+$ $CD62L^{lo}$. DCs infected with $VACV_{WR}$ (MOI = 5) for 2 hr prior to addition of splenocytes were used for quantification of the total anti-VACV $CD4^+$ T cell response (bottom right, "$VACV^+$ DCs"). Background levels were determined with uninfected DCs ("neg"). Low-frequency A28-specific response was only detectable by ELISPOT (not shown). Results are representative of three (A) and four (B) independent experiments.

responses to VACV infection, we increased the available $CD4^+$ T cell help by immunizing mice with the VACV $I1_{21-35}$ MHC II epitope (one of the first epitopes identified from a VACV virion protein), infecting the mice with VACV, and finally monitoring the subsequent antiviral antibody responses. Vaccinating with $I1_{21-35}$ MHC II epitope resulted in a strong 10-fold increase in the total VACV antibody response, as measured by a standard VACV ELISA (Figure 2A). Unexpectedly, virus neutralizing antibody titers were unimproved in VACV-infected mice preimmunized with $I1_{21-35}$ when compared to unprimed mice (Figure 2B). Although I1 is a viral virion core protein and therefore not itself a neutralizing antibody target, II-specific $CD4^+$ T cells were expected to provide intermolecular help to all B cells specific for VACV viral particle proteins and thereby boost neutralizing antibody titers (Janeway et al., 2005). Surprisingly, when serum samples were probed for the detailed antigen specificities of the antibody response with vaccinia protein microarrays, we found the increased antibody response was exclusively against I1 and not other VACV proteins (Figures 2C–2H). IgG specific for virion core protein I1 was increased 1930% ($p < 0.0004$) (Figure 2E). A10 is a second major core protein present along with I1 in VACV virions, and A10 is a target of the antiviral IgG response in infected B6 mice (see below). Nevertheless the strength of the anti-A10 IgG response was unaltered in I1 MHC II epitope vaccinated mice (Figure 2F). The IgG responses to two vaccinia surface membrane protein virion components, D8 and H3, also exhibited no improvement (Figures 2G and 2H). Selective increase in anti-I1 IgG was still observed at a memory time point (Figure 2I). Thus, antigen-specific $CD4^+$ T cells appeared to provide help only to B cells of paired protein specificity, not virion specificity.

Antibody Response Selectivity Driven by Antigen-Specific $CD4^+$ T Cells

To directly establish the role of antigen-specific $CD4^+$ T cells in the selective targeting of individual viral virion proteins for antibody responses, we performed $CD4^+$ T cell adoptive-transfer experiments. $CD4^+$ T cells were purified from donor mice immunized with $I1_{21-35}$ MHC II epitope, and cells were transferred into unimmunized recipient mice. Recipient mice were infected with VACV and examined for VACV antibody responses. Mice receiving primed $CD4^+$ T cells responded with a dramatic increase in anti-I1 IgG (1210% enhancement, $p < 0.0001$) (Figures 3A–3C), whereas the antibody responses to other viral virion protein specificities remained unchanged (Figures 3A, 3B, 3D, and 3E), starkly ignorant of the $CD4^+$ T cells transferred from the VACV $I1_{21-35}$-vaccinated mice.

This observation of paired targeting by the CD4 T cell and antibody response indicates that $CD4^+$ T cell help is preferentially provided to B cells with the identical protein specificity, even though the viral particle exists as a solid physical structure assembled from greater than 75 distinct viral protein components (Chung et al., 2006; Condit et al., 2006; Moss, 2001; Resch et al., 2006) and this structure is expected to function as a unified immunological target for B cells and B cell-T cell (B-T) interaction (Janeway et al., 2005; Milich et al., 1987; Russell and Liew, 1979; Scherle and Gerhard, 1986). Naked unwrapped viral nucleoprotein core particles are released from dying infected cells and would be expected to function in the same manner.

To determine whether these I1 core protein results were generalizable, we tested the properties of several additional VACV $CD4^+$ T cell responses shown in Figure 1. We chose MHC II epitopes from H3 and D8 because these major virion surface

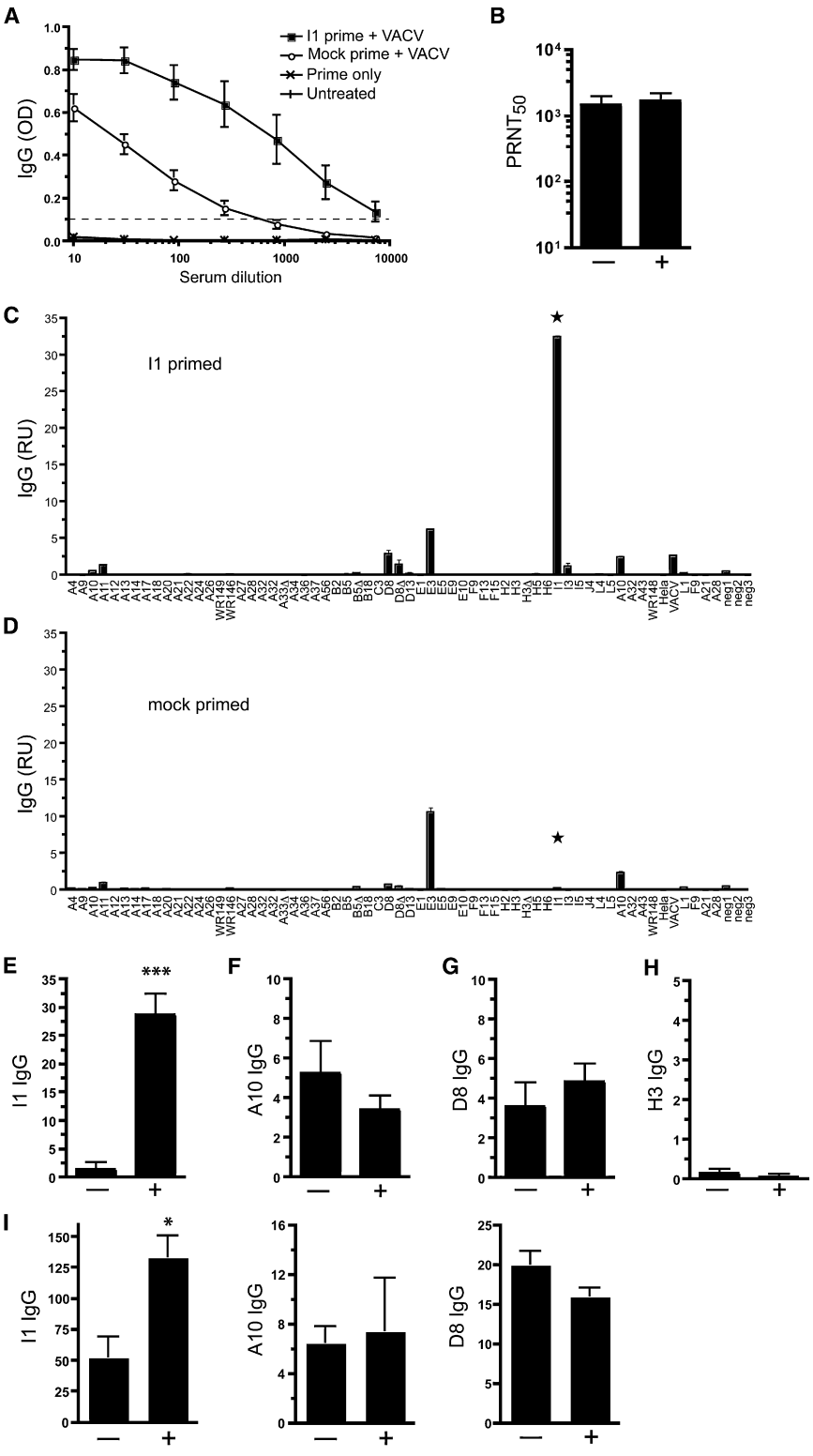

Figure 2. Selective Protein-Specific CD4+ T Cell Help to B Cells after VACV Infection

Mice peptide vaccinated with VACV $I1_{21-35}$ MHC II epitope were then infected with vaccinia virus. (A) shows Vaccinia-specific IgG responses in VACV-infected mice primed with adjuvant alone ("mock prime," open circles), I1-primed mice subsequently infected with VACV (squares), I1-primed only mice ("X" symbol), and uninfected control mice ("I" symbol) were measured by ELISA. n = 4/group. Error bars represent ± SEM. As shown in (B), virus neutralizing antibody titers ($PRNT_{50}$) were measured in I1-primed and unprimed mice ($p > > 0.05$). $I1_{21-35}$ MHC II peptide-primed mice ("+," n = 4) and mock-primed mice ("−," adjuvant-only prime; n = 4) were tested after VACV infection. Shown is mean ± SEM. (C)–(I) show results with sets of VACV proteins were synthesized and printed in microarray format for generation of VACV proteome arrays that could be probed with serum samples (see Experimental Procedures). VACV proteome microarrays were probed with serum day 7 after VACV infection from mice primed with $I1_{21-35}$ MHC II epitope or mock primed. (C) and (D) show IgG responses of individual representative mice (C) primed with $I1_{21-35}$ MHC II epitope or (D) mock primed. Stars indicate the anti-I1 signal. Error bars indicate range of replicates. (E)–(H) show antibody responses to individual vaccinia-virus protein determinants after $VACV_{WR}$ infection in groups of $I1_{21-35}$ MHC II peptide-primed mice ("+," n = 4) and mock-primed mice ("−," adjuvant-only prime. n = 4). Quantitation of anti-I1 IgG ([E], $p < 0.0004$), anti-A10 IgG ([F], $p >> 0.05$), anti-D8 IgG ([G], $p >> 0.05$), and anti-H3 IgG ([H], $p >> 0.05$) concentrations were determined. Graphs show mean ± SEM (I) IgG responses in I1-primed and not primed mice at day 30 after VACV infection. Anti-I1 IgG ($p < 0.02$), anti-A10 ($p >> 0.05$), and anti-D8 ($p >> 0.05$) responses were measured. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Data are representative of five independent experiments.

transmembrane proteins are key IgG targets (Amanna et al., 2006; Davies et al., 2005b) and are ostensibly recognized by B cells as prominent components of the surface of whole virions. In addition, we tested the vaccinia $L4_{176-190}$ MHC II epitope, representing a second major viral particle core component, to compare with the responses to the I1 core protein. Antiviral IgG responses were enhanced after VACV infection of mice primed with any one of the three new VACV MHC II epitopes (Figure 4A). VACV proteome microarray serological analysis again revealed a remarkable selectivity of the antibody response in peptide-vaccinated animals. In $H3_{272-286}$ MHC-II-epitope-primed animals, anti-H3 IgG was increased dramatically (48-fold increase, $p < 0.0001$) (Figure 4B). H3 is a known target of neutralizing antibodies (Davies et al., 2005b; Lin et al., 2000), and virus neutralizing antibody titers were selectively increased in mice with H3-specific CD4+ T cells (Figure 4C, $p < 0.02$). Anti-A10 IgG concentrations were unaltered (Figure 4B), confirming and expanding the results first obtained for I1 (Figure 2). Anti-H3 IgM concentrations were also selectively increased

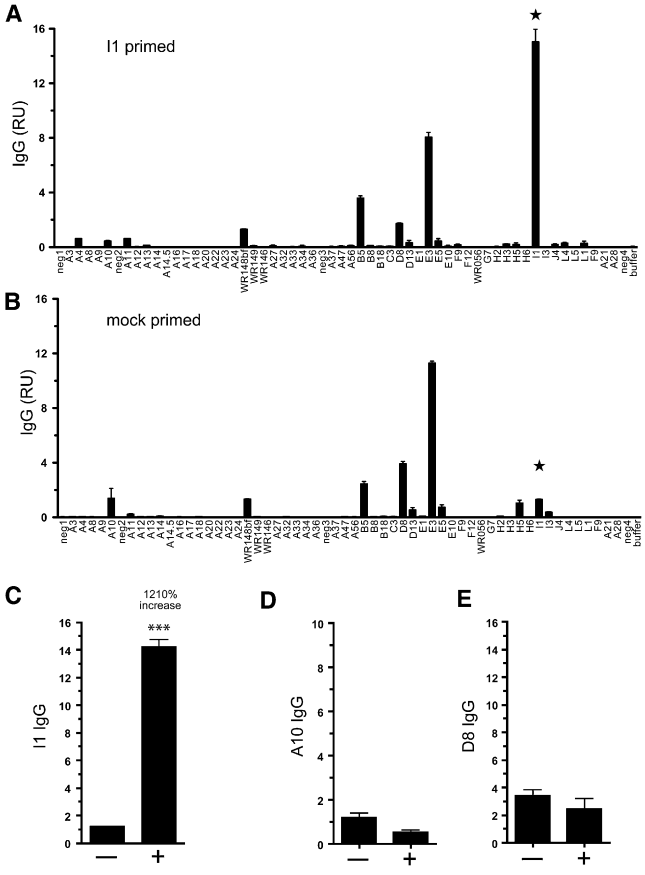

Figure 3. CD4 T Cell-Dependent MHC II-Restricted Help for VACV Antibody Response

Mice were immunized with a VACV MHC-II-binding peptide, $I1_{21-35}$. $CD4^+$ T cells were purified from donor mice and transferred to unimmunized mice. Recipient mice were then infected with VACV. (A) and (B) show quantitative analysis of IgG antibody responses to individual VACV proteins in representative mice that received (A) $I1_{21-35}$ primed $CD4^+$ T cells or (B) did not receive primed $CD4^+$ T cells. Stars indicate anti-I1 IgG. (C) shows anti-I1 IgG response in $I1_{21-35}$ MHC-II-peptide-primed $CD4^+$ T cell recipient mice ("+," $p < 0.0001$, $n = 3$; control mice "−," $n = 4$) was measured by microarray. Graphs show mean ± SEM. Anti-A10 (D) and anti-D8 (E) IgG responses measured as in (C). Data are representative of two experiments.

(Figures 4D and 4E). Particularly noteworthy, because H3 is a surface virion protein, was the observation that IgG responses against a second transmembrane surface virion protein, D8, were unaltered in H3-primed animals (Figure 4F). In contrast, anti-D8 IgG was increased substantially in $D8_{238-252}$ $CD4^+$-epitope-vaccinated mice ($p < 0.03$) (Figure 4G). The increased antibody response was again selective because anti-A10 IgG concentrations were unaltered (Figure 4G). The fourth target tested, L4, confirmed and extended the results seen above for I1, H3, and D8. Anti-L4 IgG concentrations were boosted in mice vaccinated with $L4_{176-190}$ MHC II peptide, but the remainder of the vaccinia antibody response was unaltered (Figure 4H).

Matched Immunodominant Antibody and $CD4^+$ T Cell Responses during Poxvirus Infection

The results above suggest that each antibody response need be accompanied by a matched $CD4^+$ T cell response to the same protein, as if the virion were perceived as a collection of individual protein specificities, in contrast to the expectation that the virion behaves as a unified target with promiscuous $CD4^+$ T cell help. The standard assumption has been that no direct linkage is required between B and $CD4^+$ T cell responses to pathogens, because the physical body of the pathogen (the virion or bacterium) is expected to act as a unit, a unified target for B cell binding and subsequent processing and presentation to $CD4^+$ T cells (Janeway et al., 2005). This assumption can be succinctly summarized as an "any-MHC-II-peptide-is-sufficient" model. That is, a B cell binds cognate Ag on the virion and then internalizes and processes the whole virion for Ag presentation, resulting in the B cells presenting MHC II epitopes from many different virion proteins such that interaction with a $CD4^+$ T cell specific for an epitope from any of the virion proteins will result in a cognate interaction and appropriate $CD4^+$ T cell help to the B cell (termed intermolecular help), as shown for influenza and HBV (Janeway et al., 2005; Lake and Mitchison, 1976; Russell and Liew, 1979; Scherle and Gerhard, 1986). However, our data demonstrate an unexpectedly tight linkage between the protein specificity(ies) of the $CD4^+$ T cells and the protein specificity(ies) of the VACV antibody response (intramolecular help), indicating that individual protein identities are the primary unit of immunological recognition for a large pathogen. Indeed, recognition of the physical vaccinia viral particle as a unit per se appears to be irrelevant because the recognition of I1 or H3 by $CD4^+$ T cells is predominantly nontransferable to other virion components in the context of B cell help: I1-specific $CD4^+$ T cells did not provide help to B cells of other virion protein specificities (Figures 2–4). This is of great relevance because the vast majority of potential viral antibody targets are generally considered irrelevant for protective immunity, and only virion surface proteins are relevant targets for antibody-neutralization activity. This was demonstrated by the failure to improve the neutralizing antibody response after vaccination generating I1-specific $CD4^+$ T cells (Figure 2B), in contrast to the improved neutralizing antibody titers after vaccination generating H3-specific $CD4^+$ T cells (Figure 4C).

This led us to examine whether such $CD4^+$ T cell-B cell linkage is observed in the context of the natural VACV immune response. Given the lack of predictive algorithms for identifying IA^b binding peptides (Rammensee et al., 1999; Sette et al., 1993), we identified the 14 MHC II VACV epitopes (Figure 1B) by screening an unbiased, effectively random, set of 2146 15-mer peptides sampling 31.5% of the VACV genome ORF sequences (see Experimental Procedures). These epitopes represent 26%–33% of the total anti-VACV $CD4^+$ T cell response (Figure 1B, sum of all epitopes compared to VACV-infected DC APCs), consistent with the random distribution of the 2146 peptides screened (see Experimental Procedures). Concurrent with our determination of the $CD4^+$ T cell response specificities, we identified the protein targets of the antiviral antibody response in VACV-infected B6 mice by using a series of vaccinia proteome arrays covering a total of 191 distinct genes (Figures 5A–5D and see Davies et al. [2005a] and Davies et al. [2005b]). The viral protein targets of the antibody response were determined by probing vaccinia proteome arrays with serum from >20 $VACV_{WR}$ infected animals at day 30 after infection. Definitive IgG responses were identified against 23 proteins, 17 of which were observed in the majority of infected animals (Figures 5A–5E).

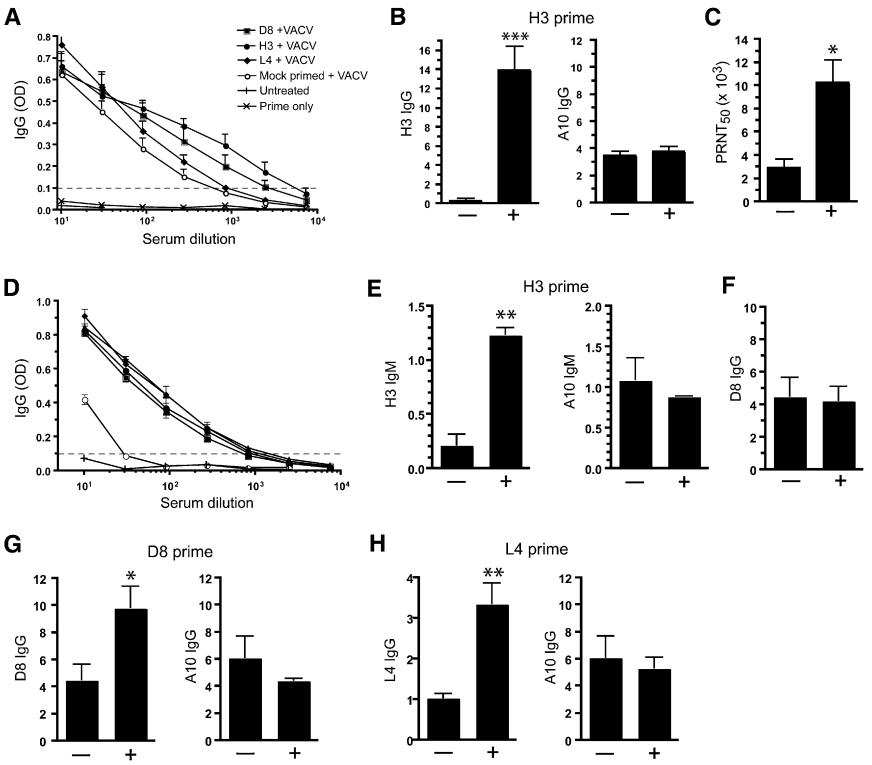

Figure 4. Highly Selective CD4+ T Cell Help to B Cells Specific for VACV Virion Components

Antiviral antibody responses were measured in mice immunized with vaccinia virus $H3_{272-286}$, $D8_{238-252}$, or $L4_{176-190}$ peptide MHC II epitopes and then infected with VACV. (A) shows vaccinia-specific IgG responses, measured by ELISA, in VACV-infected mice primed with adjuvant alone ("mock primed," open circles), H3-primed (closed circles), D8-primed (closed squares), or L4-primed (closed diamonds) mice subsequently infected with VACV. Peptide primed only mice ("X" symbol) and untreated uninfected mice (+ symbol) served as controls. n = 4/group. Error bars represent + SEM. In (B), the left panel shows anti-H3 IgG response after VACV infection in $H3_{272-286}$ MHC-II-peptide-primed mice ("+," $p < 0.0001$, n = 11, adjuvant only; "mock primed" mice, "−," n = 12; composite data from three independent experiments) are shown. The right panel shows anti-A10 concentration ($p >> 0.05$). (C) shows virus-neutralizing antibody titers ($PRNT_{50}$) in H3-primed and control mice. (D) shows vaccinia-specific IgM responses, measured by ELISA in VACV-infected (open circles), H3-primed (closed circles), I1-primed (closed squares), D8-primed (closed triangles), or L4-primed (closed diamonds) mice subsequently infected with VACV. CFA-primed-only mice (+ symbol) served as controls. n = 4/group. Mean ± SEM is shown. (E) shows anti-H3 IgM (left panel) and anti-A10 IgM responses (right panel) in $H3_{272-286}$-primed mice ($p < 0.0019$ and $p >> 0.05$). (F) shows anti-D8 IgG response in $H3_{272-286}$-primed mice ($p > > 0.05$). (G) shows anti-D8 IgG response in $D8_{238-252}$ MHC-II-peptide-primed mice (left panel: primed, "+," $p < 0.04$, n = 4; adjuvant-only "mock-primed" mice, "−," n = 4). The right panel shows anti-A10 IgG ($p >> 0.05$). (H) shows anti-L4 IgG response in $L4_{176-190}$ MHC-II-peptide-primed mice (left panel: primed, "+," n = 4; adjuvant-only "mock-primed" mice, "−," n = 4). The right panel shows anti-A10 IgG response ($p >> 0.05$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Data are representative of three to five independent experiments.

Extensive overlap was observed between the antigens recognized by the CD4+ T cells and the antibodies. Six of the VACV-protein antigens exhibited matched antibody and CD4+ T cell responses (Figure 5E), representing 46% of the known CD4+ T cell targets (6 of 13) and 26% of the antibody targets (6 of 23). Given that VACV contains greater than 200 genes, this concurrence was striking. Statistical analysis confirmed that this linkage was highly unlikely to occur by chance ($p < 0.0009$, Fisher's exact test). In contrast, no statistically significant linkage was observed between the CD4+ T cell and CD8+ T cell targets, indicating that these processes occur by distinct mechanisms (Moutaftsi et al., 2007).

The six paired B cell-CD4+ T cell targets represented all three major classes of viral proteins: virion surface proteins (H3, D8, and A28), virion core proteins (I1 and L4), and nonstructural proteins expressed within infected cells (D13). Also of note, the CD4+ T cell target antigens not matched by an antibody response were almost all (six of seven) viral transcription factors and replication factors (A18 transcription factor, A20 DNA polymerase cofactor, A24 RNA polymerase subunit, E1 polyA polymerase, and J4 RNA polymerase subunit) (Figure 5E). We speculate viral replication factors may be poor B cell antigens because of low concentration or intracellular expression. This is consistent with a model in which an antigen-specific B cell response is dependent on a CD4 T cell response of matched specificity but not vice versa.

Prediction of Antiviral CD4+ T Cell Responses on the Basis of Antibody Specificities

Prediction of CD4+ T cell response epitope specificities is difficult because of class II binding motif degeneracy and other considerations (Rammensee et al., 1999; Sette et al., 1993). Furthermore, those difficulties are exacerbated by the sheer size of large pathogens such as poxviruses. Given that our data show the VACV virion antibody responses are restricted by protein matched CD4+ T cell help (Figures 2–4), our model suggests we should be able to predict additional VACV CD4+ T cell targets by observing viral antibody target specificities. To test this hypothesis, VACV IgG targets B5, A4, A27, B2, and A33 were selected on the basis that CD4+ T cell responses had not been detected to these targets in the first round of random screening (Figure 1B and Experimental Procedures; see also Moutaftsi et al. [2007]), but we now predicted that, for each antibody target, the antibody response was predicated on a matched CD4+ T cell response (Figure 5E). As negative controls, two virion proteins, A9 and D3, that are not IgG targets were also selected. Overlapping peptides representing the entirety of each protein were screened with IFNγ ELISPOT for detection of the presence of epitope-specific CD4+ T cell responses in VACV-infected B6 mice. Responses were then confirmed by intracellular cytokine staining of CD4+ T cells from VACV-infected mice (Figure 6A). CD4+ T cell responses were identified against four of the five B cell targets tested: B5, B2, A4, and A33 (Figure 6A), thereby

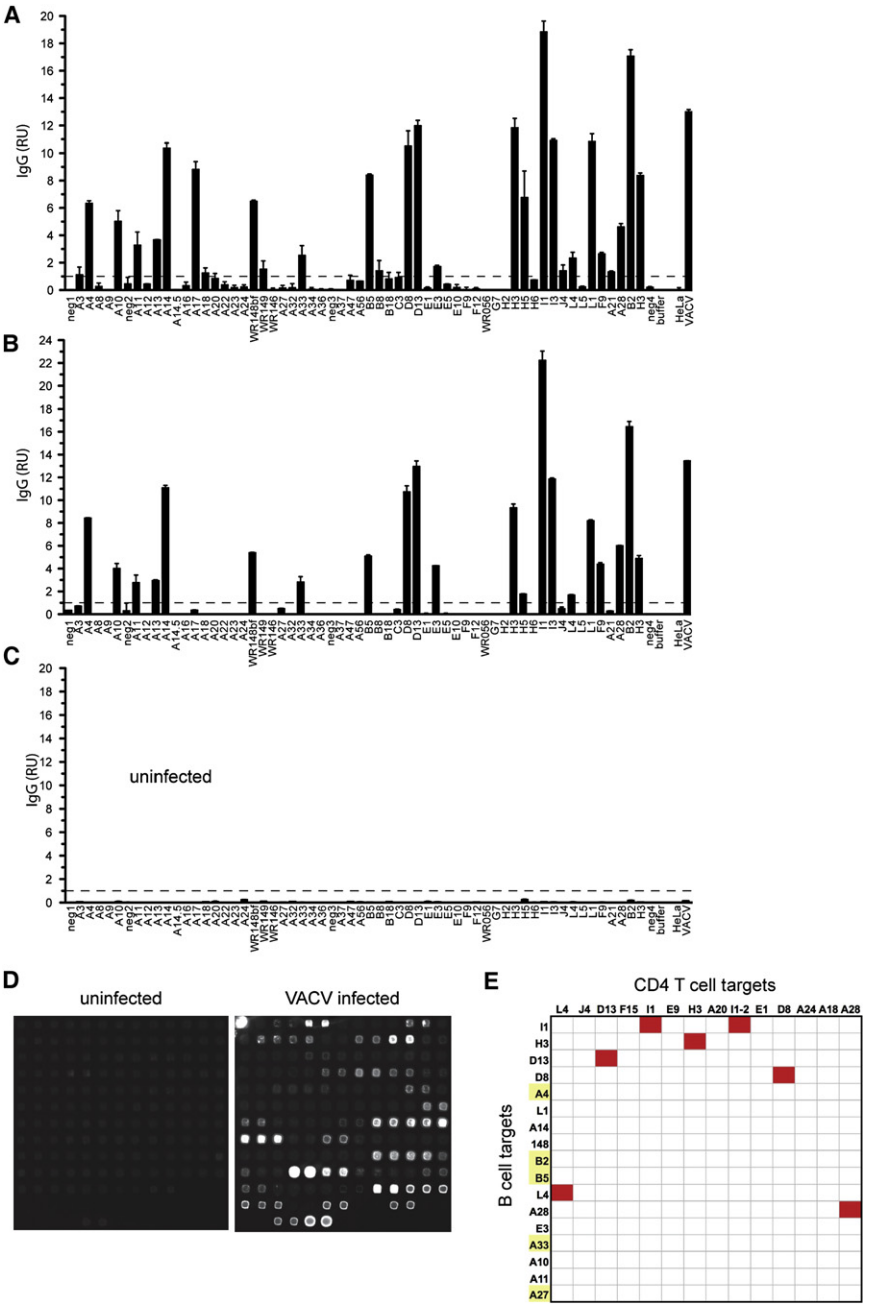

Figure 5. Interrelationship between Anti-VACV CD4+ T Cell and Antibody Responses in Virus-Infected Mice

(A–C) Quantitative analysis of IgG antibody responses to individual VACV proteins in (A and B) two representative VACV-infected B6 mice and one (C) uninfected mouse, as measured by proteomic microarray (RU, relative fluorescence units). Stringent limit of detection is indicated by dashed line. Panels are representative of >20 animals. Graphs show mean ± range.

(D) Representative immunofluorescence microarray scan of VACV-protein microarray probed with sera from an uninfected mouse versus a VACV-infected mouse. Each VACV protein is presented as duplicate spots.

(E) Tabulation of interrelationship between the antiviral CD4+ T cell targets (columns) and antibody targets (rows). Matched CD4+ T cell and antibody specificities are indicated in red. Specificities are ranked roughly in descending order on the basis of strength of response (T cell targets, left to right; antibody targets, top to bottom). A total of 17 IgG targets were identified in the majority of infected mice (shown), and variable IgG responses were also seen to minor antigens F9, I3, A56, A17, A13, and WR149 in some infected mice (not shown, but included in the statistical analysis to be conservative). B cell specificities subsequently selected for prediction of CD4 T cell responses are highlighted in yellow.

raising our total number of identified VACV CD4+ T cell epitopes to 18. No CD4+ T cell responses were detected to control ORFs A9 and D3. Strikingly, the response to $B2_{46-60}$ was the strongest CD4+ T cell response of all 18 epitopes identified, and the $B5_{46-60}$ epitope was the third strongest response overall (Figure 6A, compared with Figure 1B. We tested all peptides concurrently to allow for direct quantitative comparisons, ranked in Figure 6B). The four new epitopes accounted for 22% of the total VACV-specific CD4+ T cells in infected mice, compared with 26% accounted for by the initially identified 14 epitopes. Taking into consideration that 211 peptides were tested from the five anti-VACV target proteins in the new selective screen versus 2146 random peptides in the original screen, we found that CD4+ T cell target identification via linkage prediction based on serology represented an 830% increase in predictive power over random screening of peptides. This efficient strategy for CD4+ T cell epitope identification has obvious practical benefits for studying other complex pathogens of interest.

Role in Protective Immunity In Vivo

Having predicted and identified a CD4+ T cell response to B5, we then showed that B5-specific CD4+ T cells provide selective help to B5-specific B cells. This is of interest not only to confirm the prediction but also because B5 is a important membrane protein and neutralizing antibody target on the second form of VACV viral particle, extracellular enveloped virion (EV), which has a second outer-membrane layer, with distinct membrane proteins, covering an inner membrane and virion core that is identical to the much more abundant MV (mature virion) viral particle form (which includes H3 and D8 as surface-membrane proteins). Mice primed to generate a B5-specific CD4+ T cell response and then infected with VACV develop an enhanced B5 antibody response (Figure 7A, 4-fold increase, $p < 0.04$). Normal VACV antigen preparations are dominated by MV virion antigens and contain only trace amounts of B5 (determined with B5 monoclonal antibody, data not shown), and therefore VACV MV antigen ELISA was an efficient test for demonstrating that the B5

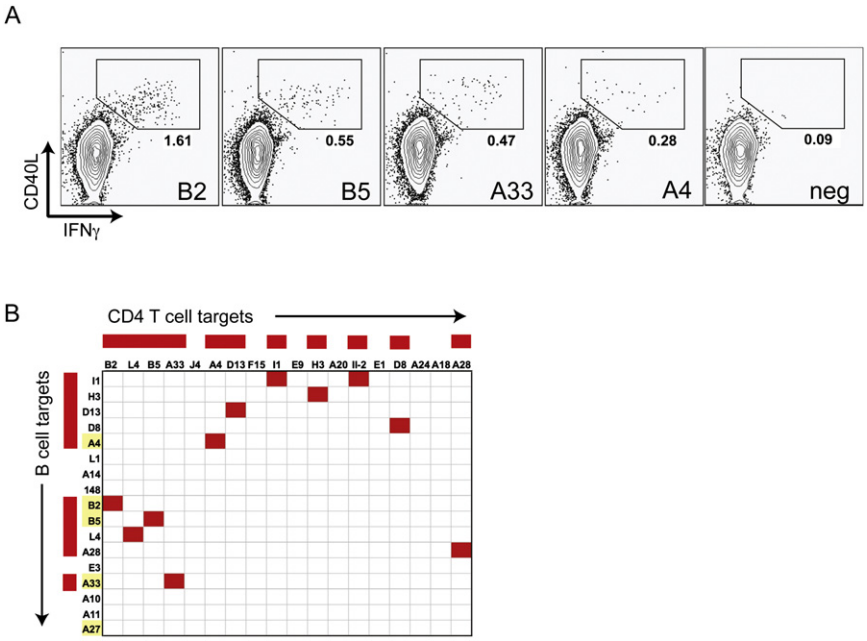

Figure 6. Utilization of Antibody Specificities to Predict New Vaccinia Virus Protein Targets of CD4 T Cell Responses

(A) IFNγ and CD40L intracellular staining for VACV $B2_{46-60}$-, $B5_{46-60}$-, $A33_{116-130}$-, and $A4_{66-80}$-specific $CD4^+$ T cells at day 10 after VACV infection. Graphs are of gated $CD4^+$ $CD62L^{lo}$ lymphocytes. "Neg" refers to no peptide control. As negative controls, two virion proteins, A9 and D3, that are not IgG targets were also selected. No $CD4^+$ T cell responses were detected to control ORFs A9 and D3 (data not shown).

(B) Tabulation of the full set of discovered interrelationships between the antiviral $CD4^+$ T cell targets (columns) and antibody targets (rows). Eleven of eighteen $CD4^+$ T cell responses (highlighted in red) are matched by IgG responses to the same smallpox vaccine virus protein. Specificities are ranked and marked as described above. Data are representative of five independent experiments.

$CD4^+$ T cells only help B5-specific B cells because the overall anti-VACV MV IgG response was not enhanced in mice with pre-existing B5-specific $CD4^+$ T cells (Figure 7A).

Knowing that B5 and H3 are both targets of protective neutralizing antibody responses (Benhnia et al., 2008; Chen et al., 2006; Davies et al., 2005a; Lustig et al., 2005), we could test the biological relevance of the T-B linkage in vivo in a lethal poxvirus challenge system. Mice were immunized with $H3_{272-286}$ or $B5_{46-60}$ MHC II peptide after the standard immunizations done previously in this study, such that they possessed B5-specific CD4s or H3-specific CD4s prior to intranasal challenge with 1×10^4 PFU $VACV_{WR}$ (~1 LD_{50}). Primed mice possessing B5 CD4s or H3 CD4s were significantly protected from morbidity (weight loss) after $VACV_{WR}$ infection compared to control adjuvant-primed mice (Figure 7B, $p < 0.007$, $p < 0.02$), and primed mice possessing I1 CD4s did not exhibit protection ($p >> 0.05$, data not shown). This protection was dependent on the enhanced neutralizing antibody response because the protection mediated by the B5- and H3-specific CD4s was completely lost in the absence of B cells, measured either by morbidity (Figure 7C) or mortality (Figure 7D; B5, $p < 0.02$; H3; $p < 0.0001$) after $VACV_{WR}$ intranasal challenge. Thus, matched CD4 T cell responses to neutralizing antbody targets are necessary for effective neutralizing antibody responses and protective immunity to VACV.

DISCUSSION

Three distinct lines of evidence demonstrate the deterministic linkage of B cell and $CD4^+$ T cell specificities to this large virus: (1) MHC II peptide vaccination results in highly selective $CD4^+$ T cell help to matched B cells specific for the same viral virion protein, (2) a strong overall correlation was found between $CD4^+$ T cell targets and antibody targets in vivo ($p < 0.0009$), and (3) new MHC II epitopes recognized by antiviral $CD4^+$ T cells were efficiently predicted on the basis of this model (830% increase in predictive power). In total, 11 of the 18 $CD4^+$ T cell responses we identified were matched by a paired antibody response to the same viral protein, including all of the top five viral protein IgG targets. Our results reveal an unexpectedly tight linkage between the $CD4^+$ T cell and antibody response specificities for VACV—the first large viral pathogen examined in this manner. Furthermore, we show that this is important for the generation of neutralizing antibodies and in vivo protective immunity. Intermolecular help has been a well-accepted viral immunology model for 20 years, on the basis of data from influenza-virus studies (Russell and Liew, 1979; Scherle and Gerhard, 1986) and corroborating evidence from hepatitis B virus (HBV) (Milich et al., 1987). However, putative whole VACV viral particle uptake and antigen presentation is not detectable, as measured by in vivo $CD4^+$ T cell help.

We consider that there are two plausible reasons for why whole VACV viral particle uptake and presentation is not observed, and we consider that there are three feasible mechanisms for how the virus-specific B cells are acquiring antigen and T cell help. The first plausible reason for the failure of whole VACV viral particle uptake and antigen presentation is a size restriction. There is a substantial size difference between the experimental pathogens being considered. VACV virions are large (360 nm diameter) (Cyrklaff et al., 2005; Roos et al., 1996), whereas flu virions are small (80 nm [Knipe and Howley, 2001]), and HBV particles are smaller still (20–45nm [Knipe and Howley, 2001]). Antigen-specific uptake by B cells is via BCR-mediated endocytosis, and endocytic vesicles are only 50–150 nm in diameter (Goldstein et al., 1979; Lodish et al., 2003; West et al., 1994). This may result in size exclusion at the level of cellular uptake and would be a restriction generally applicable to large pathogens, including large viruses, bacteria, and parasites. A second plausible reason for failure to observe whole VACV viral particle uptake and presentation could be that viral particles are not a meaningful source of antigen, either because of limiting numbers of virions produced or limited accessibility to B cells. It may be that VACV infection, although quite robust, does not reach the very

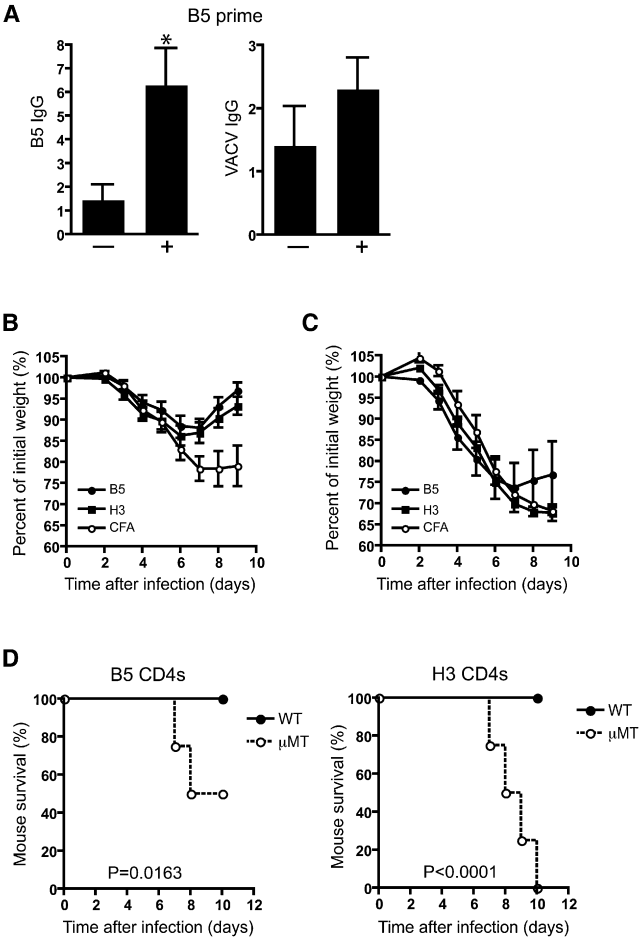

Figure 7. Protective Immunity Induction In Vivo

(A) Antibody responses to B5 (left) and VACV MV (right) proteins after $VACV_{WR}$ infection of $B5_{46-60}$ MHC-II-peptide-primed mice ("+") and mock-primed mice ("−," adjuvant-only prime).

(B) Weight loss in B6 mice infected intranasally with $VACV_{WR}$. Groups were primed for generation of B5-specific $CD4^+$ T cells (closed circles, n = 8) or H3-specific $CD4^+$ T cells (squares, n = 8) or primed with CFA adjuvant alone (n = 8) prior to viral challenge.

(C) Weight loss in B cell-deficient mice (*μMT*) infected intranasally with $VACV_{WR}$. This experiment done concurrently with that of (B). n = 4 per group. Graphs show mean ± SEM.

(D) Survival curves in B5 $CD4^+$ T cell primed (left) and H3 $CD4^+$ T cell-primed (right) C57BL/6 ("WT") and B cell-deficient mice after $VACV_{WR}$ challenge. Data are representative of two independent experiments.

high amounts of viral particle production obtained during some other infections (For example, HBV infection results in systemic serum virion levels in excess of 10^9 per ml, whereas VACV is normally undetectable [$<10^2$ per ml] in serum [Briody, 1959; Hollinger and Liang, 2001].). This scenario would then be applicable to a wide range of infections that result in midgrade physiological concentrations of pathogen. Alternatively, the absolute number of viral particles may not be the relevant parameter, but instead accessibility of antigen to B cells is the key parameter. This issue has been highlighted by several recent studies examining the impact of lymph-node architecture on antigen acquisition by B cells. Large multimolecular particles such as viruses (Hickman et al., 2008; Junt et al., 2007), bacteria (Carrasco and Batista, 2007), or antigens or comparable size (Carrasco and Batista, 2007; Phan et al., 2007) are excluded from diffusion into lymph nodes. Viruses and other particulate antigens traffic through lymph and are captured by macrophages that bridge the subcapsular sinus and the underlying B cell follicle. The particles are then transferred to B cells that migrate deep into the follicle to the T-B boundary (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007), where the B cells presumably initiate interactions with $CD4^+$ T cells. However, although VACV is excluded from freely diffusing through follicles (Hickman et al., 2008; Junt et al., 2007), and VACV replication is highest in ovaries (Briody, 1959; Xu et al., 2004), it is also known that VACV directly infects lymph node and spleen cells (Briody, 1959; Hickman et al., 2008) and has considerable access to cells in the lymph-node medulla (Junt et al., 2007). Furthermore, DCs can retain intact microbes (Balazs et al., 2002; Kwon et al., 2002; Macpherson and Uhr, 2004) and transfer antigens to B cells (Qi et al., 2006; Wykes et al., 1998), suggesting multiple different routes by which whole virions may encounter B cells.

Given those possible reasons why whole VACV virion uptake and antigen presentation is not occurring at detectable levels, there are three feasible mechanisms for how the B cell antigen uptake and presentation is occurring during a VACV infection. The first and simplest model is that VACV virions are not involved (for reasons described above) and free individual viral proteins are the physiological B cell antigens. Data are available that support this model. Soluble proteins can be rapidly trafficked through lymph and be acquired by lymph-node-resident B cells (Pape et al., 2007). This has also been observed in the context of a viral infection (VSV [Junt et al., 2007]). The appeal of this model is only weakened by the consideration that monomeric soluble antigens would fail to induce BCR crosslinking and signaling. This lack of BCR signaling would fail to enrich for antibody responses to virion surface proteins presented in their appropriate conformation. Because virion surface proteins are generally the only physiologically relevant target antigens for neutralizing antibody responses, and virion surface protein-specific B cell responses are efficiently induced by the highly multimeric nature of virions via extensive BCR crosslinking upon interaction with a virus-specific B cell (Bachmann and Zinkernagel, 1997), whole-virion binding by virus-specific B cells is the only opportunity for the immune system to selectively enrich for B cell responses to neutralizing antibody targets. One could argue for an intermediate level of macromolecular structure (subvirion fragments or particles) such that sufficient copies of antigen are available for BCR crosslinking (which may be occurring in the context of VSV infection, as observed in Supplemental Figure 6 of Junt et al. [2007]), but this is an unlikely solution for large viruses with complex virion protein mixtures because the macromolecular structures would have to be of a single-protein species to fit the observation that $CD4^+$ T cell help to VACV B cells is highly protein specific. However, a second potential mechanism would allow immunologic utilization of the information contained in the multimeric nature of the virion: If B cells bind to whole virions in vivo but are unable to internalize the large particle, the B cells may pinch off cognate antigen after forming an immunological synapse with the target membrane (in this case, the virion surface-membrane or infected cell). B cells are known

to be able to extract antigen from target cell membranes (Batista et al., 2001) or planar membrane surfaces (Fleire et al., 2006) in a BCR-mediated process, and the acquired antigen induces B cell activation and is efficiently processed and presented to cognate T cells (Batista et al., 2001; Fleire et al., 2006). Concentration of membrane-bound antigen by formation of an immunological synapse and subsequent pinching of that antigen would provide a mechanism both to enhance recognition of potential neutralizing antibody targets via increased BCR mediated stimulation and a mechanism for selective $CD4^+$ T cell help. A third potential mechanism for selective B-T interactions would be preferential protection of cognate antigen by the BCR during transport to intracellular antigen-processing compartments (Watts, 1997). Further studies are required to discriminate between these mechanisms. Initial attempts to elucidate molecular mechanism were inconclusive because of the low frequencies of antigen-specific B cells in vivo (data not shown), and therefore BCR transgenics will be required to address this issue. Panels of VACV hybridomas have now been generated and characterized (unpublished data), and BCR cloning and transgenic production is underway. It will also be important to test the prediction of B-T linkage for additional large pathogens and the general power of serological analysis to greatly enhance prediction and identification of novel $CD4^+$ T cell epitopes for other large pathogens of interest. It is intriguing to speculate that these findings may also relate to the mechanisms underlying B-T linkage to surface membrane proteins in cancer immunity and autoimmunity (e.g., myasthenia gravis), for which the antigen is present is the context of a whole cell.

The data presented herein demonstrate that the cellular mechanism driving the linkage of $CD4^+$ T cell and antibody specificities is a B cell requirement for intramolecular protein-specific $CD4^+$ T cell help. As such, MHC restriction at the protein level is a key event for humoral immune responses to VACV because individual protein identities are the primary unit of immunological recognition. This is a powerful principle; we have shown that it impacts protective immunity and can also be used to predict the presence of novel pathogen-specific $CD4^+$ T cell responses. These findings from the smallpox vaccine virus are relevant for understanding the nature of B cell antigen presentation to $CD4^+$ T cells and nonself recognition, the prediction of MHC II epitopes, and vaccine development against complex pathogens. Because the smallpox vaccine is the only vaccine to result in eradication of a disease from the human population, immunological results with this virus must be taken quite seriously.

EXPERIMENTAL PROCEDURES

Mouse Procedures and Viral Infections

C57BL/6J (B6), MHC class $II^{-/-}$ (C57BL/6J Ia^b-$Ea^{-/-}$), and B cell-deficient (C57BL/6J *μMT*) mice were purchased from the Jackson Laboratory and bred in house. $VACV_{WR}$ (Western Reserve strain) stocks were grown on HeLa cells, infecting at a multiplicity of infection of 0.5 (MOI = 0.5). Cells were harvested at 60 hr, and virus was isolated by rapid freeze-thawing the cell pellet 3× in a volume of 2.3 ml RPMI + 1% FCS per T175 flask. Cell debris was removed by centrifugation. Clarified supernatant was frozen at –80°C as virus stock (Davies et al., 2005b). Stocks were titered on VeroE6 cells. For all experiments except Figures 6D–6F, mice were infected with $VACV_{WR}$ by bilateral intraperitoneal (i.p.) injections of 2×10^5 or 2×10^6 total PFU total with standard $VACV_{WR}$ stocks. Replicates of experiments in Figures 1, 2, 4, and 5 were also performed with purified $VACV_{WR}$ for infections; no differences in the results were observed. Purified $VACV_{WR}$ stocks were made by centrifuging standard $VACV_{WR}$ stock through a 36% sucrose cushion in PBS plus 5 mM $MgCl_2$ and resuspension of the virion pellet in RPMI + 1% FCS. For peptide immunizations, 30 μg peptide (or PBS control) was emulsified in CFA. Subcutaneous injections were done dorsal to the base of the tail; subcutaneous injections for some repeat experiments were done between the scapula. $VACV_{WR}$ i.p. infections were done 11–13 days after peptide priming. For adoptive transfers, untouched $SMtg^+$ CD4 T cells were magnetically purified from spleen and lymph-node preparations (MACS Miltenyi). A total of 50×10^6 $CD4^+$ T cells were transferred per mouse, resulting in approximately one-half mouse equivalent of donor $CD4^+$ T cells after take (e.g., 5×10^6 donor CD4 T cells/spleen). For viral challenge experiments (Figures 6D–6F), 8-week-old female mice were infected intranasally with 1×10^4 PFU $VACV_{WR}$. Virus was placed in a volume of 10 μl on the nares of lightly sedated mice and subsequently inhaled by the mice. Mice were weighed daily to track disease progression. Dose-titration experiments established 1×10^4 PFU $VACV_{WR}$ to be ~1 LD_{50} in 8-week-old female C57BL/6 mice (unpublished data). All animal experiments were conducted in accordance with approved animal protocols.

ELISA

VACV ELISAs were done as described (Davies et al., 2005b), with the additional use in Figure 1 of standard curves with mouse IgG (Southern Biotech) in anti-mouse Ig (goat anti-mouse IgM + IgG + IgA, Caltag)-coated wells. B5 ELISA used recombinant B5.

Protein Arrays and Analysis

We used 16-pad nitrocellulose FAST slides (Whatman) for protein microarray printing. Proteins were printed at Scripps (TSRI) with a custom arrayer with 100 μm pins built by Robotic Labware Design (RLD). Humidity was maintained at 40%–60% during printing. Microarray slides were subsequently dried and stored in a desiccator at −80°C. Vaccinia genes were cloned into pXi (pNHisCHA derived [Davies et al., 2005b]) and sequenced prior to protein synthesis with the Roche RTS *E. coli* in vitro-coupled transcription and translation expression system. RTS reactions without plasmid were used as negative controls. Expression was confirmed by dot blotting, immunoblotting, or microarray probing for His tags. Whole-virus antigen was printed with 10-fold concentrated, PBS buffer-exchanged, psoralen inactivated (Davies et al., 2005b) standard $VACV_{WR}$ stock. Uninfected HeLa cell lysate processed identically served as negative control (Crotty et al., 2003). Purified His-tagged H3L protein (Davies et al., 2005b) was printed at 100 μg/ml. *E. coli* produced L1, F9, A21, and A28 were purified and refolded under oxidizing conditions for generation of natively folded protein with appropriate disulfide-bond formation (Su et al., 2005). Secreted B5R was produced with a baculovirus expression vector in S2 insect cells and subsequently purified with an N-terminal His tag. All antigens were resuspended in 0.02% Tween-20 prior to printing.

Mouse serum samples were initially screened with a large protein microarray covering 185 VACV proteins (Davies et al., 2005b). Because only a minority of VACV antigens elicit antibody responses in mice, humans, hyperimmune rabbits, or primates (Davies et al. [2005a], Davies et al. [2007], and D.D., S.C., P.F., unpublished data), the majority of experiments done in this study were done with a compact microarray (Figure 5) consisting of 55 proteins identified as an antibody target in any species, as well as all known virion surface proteins (as of early 2005). This strategy allowed for an 8-fold increase in the numbers of replicate experiments (16 pad microarray slide versus two pad microarray slide). In addition, after the identification of VACV CD4 T cell targets (Figure 1), we retested CD4 T cell target proteins without known IgG responses (J4, F15, A20, E1, E9, A24, and A18) in a new set of microarrays to determine whether any IgG targets had been missed, and no new IgG targets were detected. In total, using several generations of microarrays, we tested 191 VACV proteins for IgG responses, out of 218 total annotated $VACV_{WR}$ genes.

We used proteome microarrays to detect antibodies by techniques comparable to a fluorescence ELISA on a microscale. After blocking arrays (Protein Array Blocking Solution, Whatman), microarrays were probed with 1:50 mouse serum diluted in array-blocking solution preadsorbed for 30 min with 10% clarified *E. coli* lysate. (Clarified *E. coli* lysate was prepared with 100 mg/ml DH5α in PBS, sonicated, and centrifuged at 6000 g. Lysate was then stored at −80°C.) After 2 hr of incubation, extensive washing was done with PBS + 0.05% Tween-20, and secondary antibody (Cy3 labeled goat anti-mouse

gamma chain Fc region specific immunoglobulin, Jackson Immunoresearch) at 1:50 in array-blocking solution was added for 1 hr. Cy5-labeled goat anti-mouse IgM Fc-specific $F(ab)_2$ immunoglobulin was also used in some experiments. Arrays were then washed extensively with PBS + 0.05% Tween-20 and PBS alone and then spun dry. Arrays were scanned on an Axon 400B GenePix (Molecular Dynamics), and data were acquired with GenePix Pro 5.1. Total 532 nm fluorescence intensity (TFI_{532}) of each spot was the signal strength. Background signal was subtracted out with relevant matched control samples (e.g., RTS translation reaction without plasmid), and background-subtracted signal was converted to the final IgG relative units (RU) via 10^{-6} transformation. Data are plotted as the average of duplicate protein prints (spots), with the full range shown as the error bar (e.g., Figures 5A–5C). Stringent signal thresholds were established as 1 RU above background, and this unit was greater than 10× the background observed in uninfected animals for individual protein antigens (Figure 5). With these experimental conditions, IgG signals detected by protein microarray are linear and correlate tightly with signals observed by conventional endpoint dilution ELISA.

Neutralization Assays

VeroE6 cells were seeded into 24-well Costar plates (Corning) and used within 24 hr of reaching 90% confluency. Mouse serum samples were collected by retro-orbital bleed from B6 mice at various time points after infection. All serum samples were heat-inactivated prior to use (56°C, 30 min). Diluted sera were incubated in an equal volume of sonicated sucrose gradient purified $VACV_{WR}$ (10^4 PFU/ml) overnight at 37°C, 5% CO_2 (Newman et al., 2003). Negative control sera from untreated mice were also done at the same conditions. The medium from 24-well plate was aspirated, and 100 μl of virus/serum mixture was added to each well and left to adsorb for 60 min at 37°C with periodic swirling. The infected VeroE6 cells were rinsed with warm PBS. One ml of complete D-10 medium (DMEM + 15% FBS + pen/strep + L-glut) was then added, and the plates were incubated for 40–50 hr. Medium was then aspirated, cells were fixed and stained in one step with 0.1% crystal violet in 20% ethanol, and plaques were quantified over white-light transillumination. Fifty percent of plaque reduction neutralization titer ($PRNT_{50}$) was defined as the furthest dilution of each sample to neutralize >50% the virus according to the formula: [(PN VACV – PN sample)/PN VACV], where "PN VACV" is the average number of plaques in wells infected with $VACV_{WR}$ alone and "PN sample" is the average number of plaques for a serum sample at a given dilution.

CD4+ T Cell Assays and MHC II Epitope Screening

Initial VACV epitope screening by IFNγ ELISPOT was described (Moutaftsi et al., 2007). The 2146 15-mer peptides represented 31.5% of the VACV genome-coding sequence. These peptides were synthesized for various purposes, without consideration of antibody targets or other potential biases, such that the peptide screening library randomly queried sequences throughout the VACV amino acid sequence space. The peptides represented 199 of the 218 annotated VACV genes (18 of 19 missed ORFs are less than 80 amino acids in length). In the 2146 peptides (32,190 amino acids), 30% of total structural protein sequence and 31% of total nonstructural protein sequence were represented. The percentage of the anti-VACV $CD4^+$ T cell response identified with these peptides (26%–33%, Figure 1B) was consistent with the random distribution and overall VACV sequence coverage of the peptides (31.5%). In addition, we could retroactively examine the peptide-library contents for bias in coverage of known antibody targets, and no bias is present (30.7% amino acid coverage of antibody targets versus 30.4% coverage of other), independently confirming the unbiased nature of the peptide library used.

The overlapping peptides used in Figure 6 were synthesized as 15-mers overlapping by 5 amino acids on each end. We calculated increased epitope identification predictive power via utilization of antibody targets (Figure 6) by summing the $CD4^+$ T cell responses to each epitope of interest (Figure 1B and Figure 6A, experiments done at the same time) and then dividing by the total anti-VACV CD4 T cell response (Figure 1B). This was then divided by the number of peptides screened in each group.

Intracellular cytokine staining of splenocytes from $VACV_{WR}$-infected mice (day 7 or 10 after infection) was done by incubation of splenocytes with peptide-pulsed $CD11c^+$ DC for 1 hr prior to addition of brefeldin A. After 5 hr, cells were surface stained for CD62L and CD4 surface molecules; this was followed by intracellular staining for IFNγ and CD40L. IFNγ-producing and/or CD40L expressing cells were determined by gating lymphocytes on FSC/SSC and then gating on CD62L low/$CD4^+$ T cells. $CD11c^+$ DCs infected with $VACV_{WR}$ (MOI = 5) for 2 hr prior to addition of splenocytes were used for identification of the total anti-VACV $CD4^+$ T cell response. We generated DCs by subcutaneous implantation of Flt3L-producing B16 cells and then by harvesting $CD11c^+$ DCs from spleen at day 12 after implantation, with CD11c paramagnetic beads (MACS Miltenyi). Background level was established with $CD4^+$ T cell values in the presence of uninfected $CD11c^+$ DCs. All antibodies were purchased from eBiosciences or BD PharMingen.

Statistics

Tests were performed with Prism 4.0 (GraphPad) or VassarStats (Fisher's exact test). Statistics were done with two-tailed, unpaired Student's t test with 95% confidence bounds unless otherwise indicated. Bar graph error bars are ± 1 SEM, except for raw microarray data, which show full range (e.g., Figures 5A–5C). Arithmetic means were used for all analyses. $CD4^+$ T cell-antibody linkage (Figure 5E) was analyzed with Fisher's exact test with the null hypothesis being no increased frequency of antibody targets among the $CD4^+$ T cell target proteins. Six $CD4^+$ T cell target proteins were IgG targets; seven of them were not. Twenty-three of 191 VACV proteins screened were IgG targets. We did statistical analysis of mouse weight loss after intranasal infection with $VACV_{WR}$ (Figures 6D and 6E) by tabulating the weight nadir (or 70% for dead mice, which was the maximum weight loss before euthanization) for each mouse and then comparing each experimental group by standard two-tailed, unpaired Student's t test with 95% confidence bounds. Survival-curve significance was calculated with Kaplan-Meier statistical analysis.

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